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REMARKS

Claims 30-49 are pending in the Application, with claims 30-33, 37-39, 41-43 and 45-49 withdrawn. Claims 34-36 and 44 are sought to be amended without prejudice thereto or disclaimer thereof any subject matter contained within the previously presented versions of these claims. Support for the amended claims can be found, for example, throughout the specification and in the original claims. Applicants have not raised any issue of new matter.

I. Specification

The Examiner has objected to Applicants' specification for containing embedded hyperlinks, other form of browser-executable code and/or use of trademarks. Office Action, page 3. Applicants have amended the specification as suggested by the Examiner, and respectfully request that the objection be reconsidered and withdrawn.

II. Claim Objections

Claim 35 is objected to for being dependent on claim 30, which is non-elected. Office Action, page 3. Applicants have amended claim 35 and request that this objection be reconsidered and withdrawn.

III. Claim Rejections

A. 35 U.S.C. §112, first paragraph-- Written Description Rejection

Claims 34-36, 40 and 44 are rejected under 35 U.S.C. §112, first paragraph for allegedly "containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." Office Action, page 4.

Solely to advance prosecution, and not in acquiescence to the rejection, Applicants have amended the claims. Accordingly, Applicants believe that this rejection is moot and respectfully request that the Examiner reconsider and withdraw the rejection.

B. 35 U.S.C. §112, first paragraph-- Enablement Rejection of Claims 34-36, 40 and 44

Claims 34-36, 40 and 44 are rejected under 35 U.S.C. §112, first paragraph because allegedly "[t]he specification does not enable any person skilled in the art to which it pertains or with which it is most nearly connected, to make/use the invention commensurate in scope with . . . [the] claims." Office Action, page 6.

Solely to advance prosecution, and not in acquiescence to the rejection, Applicants have amended the claims. Accordingly, Applicants believe that this rejection is moot and respectfully request that the Examiner reconsider and withdraw the rejection.

C. 35 U.S.C. §112, first paragraph-- Enablement Rejection of Claims 36 and 40

Claims 36 and 40 are rejected under 35 U.S.C. §112, first paragraph because allegedly "the specification is devoid of any teaching that said proteins provide an effective vaccine against any disease." Office Action, pages 8-9. Applicants respectfully disagree with this rejection and respectfully request that the Examiner hold it in abeyance until all other issues have been resolved.

D. 35 U.S.C. §112, second paragraph-- Distinctness Rejection

Claims 36 is rejected under 35 U.S.C. § 112, second paragraph, as allegedly "being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention." Office Action, page 10. In particular, the Examiner asserts that

> Claim 36 is rendered vague and indefinite by the phrase "a vaccine for combating *Osteratagia ostertagi* infection." To combat an infection, there must be an infection to fight, however, a vaccine, by definition, prevents infection. Therefore, a composition for combating infection cannot be a vaccine.

Id. Applicants respectfully disagree.

The M.P.E.P. clearly states that the perspective of the skilled artisan is an important factor in determining the definiteness of a claim. *See* M.P.E.P., 8th ed., § 2173.02 (revised

October 2005). Here, the skilled artisan would readily understand that one can combat an infection by preventing its spread from one herd of cattle to another, or from one cow to other cows within a herd. Hence, one is combating an infection with a vaccine even though the infection is present. Moreover, Applicants fully disagree with the Examiner's assertion that in order to combat an infection, there must be an infection to fight. Applicants assert that the infection is being combated by, for example, preventing it from establishing itself in the host organism.

Other recitations within claims 36, 40 and 44 have also given rise to a rejection under 35 U.S.C. § 112, second paragraph, for allegedly "being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention." Office Action, page 10. Solely to advance prosecution, and not in acquiescence to the rejection, Applicants have amended these other recitations within these claims. Accordingly, Applicants believe that this rejection is moot and respectfully request that the Examiner reconsider and withdraw the rejection.

E. Rejections Under 35 U.S.C. § 102

1. Claerebout et al.

Claims 36 and 40 are rejected under 35 U.S.C. § 102(a) for allegedly being anticipated by Claerebout *et al.* Office Action, page 11. In particular, the Examiner refers to slides 4-5. *See id.* Applicants respectfully traverse the rejection.

The M.P.E.P. clearly states that "[t]o anticipate a claim, the reference must teach every element of the claim." M.P.E.P. 8th ed., § 2131 (revised October 2005). Hence, notwithstanding the availability of a reference as prior art based upon its publication date, a rejection under 35 U.S.C. § 102 cannot be set forth unless the reference describes each and every claim element.

Applicants' claims 36 and 40 are both ultimately dependant upon claim 34, which is directed to a 30 kD protein as depicted in SEQ ID NO: 10. Hence, by necessity, claims 36 and 40 each include the 30 kD protein identified by SEQ ID NO: 10 as a claim element. In contrast, Claerebout *et al.* fails to mention any 30 kD protein, much less SEQ ID NO: 10. Therefore,

Claerebout *et al.* does not anticipate Applicants' claims 36 and 40. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw the rejection.

Moreover, Claerebout *et al.* cannot be used to set forth an obviousness rejection of the claims. The M.P.E.P. states that among other requirements

> [t]o establish a *prima facie* case of obviousness, . . . there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one or ordinary skill in the art, to modify the reference or to combine reference teachings. . . . [T]he prior art reference (or references when combined) must [also] teach or suggest all the claim limitations.

M.P.E.P. 8th ed., §2143 (revised October 2005). Because there is no suggestion or motivation to modify Claerebout *et al.* or to combine it with other references, and because there are no references with which it can be combined to teach or suggest all of the claim limitations, an obviousness rejection based upon Claerebout *et al.* cannot be set forth.

2. Silverman

Claims 36 and 40 are rejected under 35 U.S.C. § 102(b) for allegedly being anticipated by Silverman (U.S. 3,395,218). Office Action, page 11. In particular, the Examiner refers to column 4, lines 6-35 and column 3, lines 16-18. *See id.* Applicants respectfully traverse the rejection.

As described above, the M.P.E.P. clearly states that "[t]o anticipate a claim, the reference must teach every element of the claim." M.P.E.P. 8th ed., § 2131 (revised October 2005). Hence, notwithstanding the availability of a reference as prior art based upon its publication date, a rejection under 35 U.S.C. § 102 cannot be set forth unless the reference describes each and every claim element.

Applicants' claims 36 and 40 are both ultimately dependant upon claim 34, which is directed to a 30 kD protein as depicted in SEQ ID NO: 10. Hence, by necessity, claims 36 and 40 each include the 30 kD protein identified by SEQ ID NO: 10 as a claim element. In contrast, Silverman fails to mention any 30 kD protein, much less SEQ ID NO: 10. Therefore, Silverman

does not anticipate Applicants' claims 36 and 40. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw the rejection.

Moreover, Silverman cannot be used to set forth an obviousness rejection of the claims. The M.P.E.P. states that among other requirements

> [t]o establish a *prima facie* case of obviousness, . . . there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one or ordinary skill in the art, to modify the reference or to combine reference teachings. . . . [T]he prior art reference (or references when combined) must [also] teach or suggest all the claim limitations.

M.P.E.P. 8th ed., §2143 (revised October 2005). Because there is no suggestion or motivation to modify Silverman or to combine it with other references, and because there are no references with which it can be combined to teach or suggest all of the claim limitations, an obviousness rejection based upon Silverman cannot be set forth.

3. Pastan et al.

Claim 44 is rejected under 35 U.S.C. § 102(b) for allegedly being anticipated by Pastan *et al.* (U.S. 6,232,086). Office Action, page 12. In particular, the Examiner refers to column 22, lines 58-66. *See id.* Applicants respectfully traverse the rejection.

As described above, the M.P.E.P. clearly states that "[t]o anticipate a claim, the reference must teach every element of the claim." M.P.E.P. 8th ed., § 2131 (revised October 2005). Hence, notwithstanding the availability of a reference as prior art based upon its publication date, a rejection under 35 U.S.C. § 102 cannot be set forth unless the reference describes each and every claim element.

Applicants' claim 44 is ultimately dependant upon claim 34, which is directed to a 30 kD protein from *Ostertagia ostertagi* as depicted in SEQ ID NO: 10. Hence, by necessity, claim 44 includes the *Ostertagia ostertagi* 30 kD protein identified by SEQ ID NO: 10 as a claim element. In contrast, Pastan *et al.* fails to mention *Ostertagia ostertagi*, much less any 30 kD *Ostertagia ostertagi* protein or SEQ ID NO: 10. Therefore, Pastan *et al.* does not anticipate Applicants'

claim 44. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw the rejection.

Moreover, Pastan *et al.* cannot be used to set forth an obviousness rejection of the claims. The M.P.E.P. states that among other requirements

> [t]o establish a *prima facie* case of obviousness, . . . there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one or ordinary skill in the art, to modify the reference or to combine reference teachings. . . . [T]he prior art reference (or references when combined) must [also] teach or suggest all the claim limitations.

M.P.E.P. 8th ed., §2143 (revised October 2005). Because there is no suggestion or motivation to modify Pastan *et al.* or to combine it with other references, and because there are no references with which it can be combined to teach or suggest all of the claim limitations, an obviousness rejection based upon Pastan *et al.* cannot be set forth.

4. Coyne

Claims 34-36 and 40 are rejected under 35 U.S.C. § 102(b) for allegedly being anticipated by Coyne (U.S. 6,017,757). Office Action, page 12. In particular, the Examiner states the following

> Coyne discloses an *Ostertagia ostertagi* protein with an approximate molecular weight of 29-33 kD (see column 25, lines 14-17). Due to the similarity in molecular weight between the protein disclosed by Coyne and the protein of the instant invention it is deemed, in the absence of evidence to the contrary, that the two proteins are the same.

Id. Applicants provide evidence herewith demonstrating that Coyne does not disclose Applicants' proteins.

Immediately following the excerpt cited by the Examiner, Coyne states the following:

> Furthermore, these Con-A binding fractions were shown to possess aminopeptidase-M activity. The significance of these data is that analogous proteins of similar molecular weights harvested from parasite intestinal cells possess both aminopeptidase-M activity and Con-A binding avidity (McMichael-Phillips et al., 1995).

See U.S. 6,017,757, column 25, lines 17-23. Hence, the 29-33 kD proteins identified by Coyne have aminopeptidase-M activity.

Aminopeptidase M is an enzyme classified as EC 3.4.11.2, and is also termed Aminopeptidase N. *See* Exhibit A. Moreover, all such aminopeptidases have several consensus sequences, as shown by Figure 3 of Knight, P. J. K. *et al., J. Biol. Chem. 270:* 17765-17770 (1995) (provided herewith as Exhibit B). Because these consensus sequences cannot be found in SEQ ID NO: 10, it is clear that SEQ ID NO: 10 does not belong to the same class of proteins to which the Coyne 29-33 kD proteins belong. Hence, Coyne does not anticipate Applicants' SEQ ID NO: 10 or claims 34-36 and 40. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw the rejection.

Moreover, Coyne cannot be used to set forth an obviousness rejection of the claims. The M.P.E.P. states that among other requirements

> [t]o establish a *prima facie* case of obviousness, . . . there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one or ordinary skill in the art, to modify the reference or to combine reference teachings. . . . [T]he prior art reference (or references when combined) must [also] teach or suggest all the claim limitations.

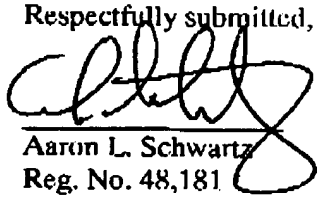
M.P.E.P. 8th ed., §2143 (revised October 2005). Because there is no suggestion or motivation to modify Coyne or to combine it with other references, and because there are no references with which it can be combined to teach or suggest all of the claim limitations, an obviousness rejection based upon Coyne cannot be set forth.

IV. Conclusion

Applicants do not believe that any other fee is due in connection with this filing. If, however, Applicants do owe any such fee(s), the Commissioner is hereby authorized to charge the fee(s) to Deposit Account No. 02-2334. In addition, if there is ever any other fee deficiency or overpayment under 37 C.F.R. §1.16 or 1.17 in connection with this patent application, the Commissioner is hereby authorized to charge such deficiency or overpayment to Deposit Account No. 02-2334.

Applicants submit that this application is in condition for allowance, and request that it be allowed. The Examiner is requested to call the Undersigned if any issues arise that can be addressed over the phone to expedite examination of this application.

Respectfully submitted,



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USSN: 10/527,771
Attorney Docket: I-2002.015 US

EXHIBIT A

NiceZyme View of ENZYME: EC 3.4.11.2

Official Name

Membrane alanyl aminopeptidase.

Alternative Name(s)

Amino-oligopeptidase.

Aminopeptidase M.

Aminopeptidase N.

Membrane alanine aminopeptidase.

Membrane aminopeptidase I.

Microsomal aminopeptidase.

Particle-bound aminopeptidase.

Peptidase E.

Reaction catalysed

Release of an N-terminal amino acid, Xaa-|-Yaa- from a peptide, amide or arylamide. Xaa is preferably Ala, but may be most amino acids including Pro (slow action). When a terminal hydrophobic residue is followed by a prolyl residue, the two may be released as an intact Xaa-Pro dipeptide

Cofactor(s)

Zinc.

Comment(s)

- Is not activated by heavy metal ions.
- Belongs to peptidase family M1.
- Formerly EC 3.4.1.2, EC 3.4.3.5 and EC 3.4.13.6.

Cross-references

PROSITE	PDOC00129
BRENDA	3.4.11.2
PUMA2	3.4.11.2
PRIAM enzyme-specific profiles	3.4.11.2
Kyoto University LIGAND chemical database	3.4.11.2
IUBMB Enzyme Nomenclature	3.4.11.2
IntEnz	3.4.11.2
MEDLINE	Find literature relating to 3.4.11.2
MetaCyc	3.4.11.2
UniProtKB/Swiss-Prot	Q9CIQ1, AMPN1_LACLA; Q48656, AMPN2_LACLA; Q10736, AMPN_ACEPA; P79098, AMPN_BOVIN; P79143, AMPN_CANFA; P37893, AMPN_CAUCR; P04825, AMPN_ECOLI; P79171, AMPN_FELCA; Q10737, AMPN_HAECC; P45274, AMPN_HAEIN; P81731, AMPN_HELAM; P15144, AMPN_HUMAN; P37896, AMPN_LACDL; Q10730, AMPN_LACHE; P37897, AMPN_LACLC; P91885, AMPN_MANSE; P97449, AMPN_MOUSE; P15145, AMPN_PIG; P91887, AMPN_PLUXY; P15541, AMPN_RABIT; P15684, AMPN_RAT; Q11010, AMPN_STRLI;

View entry in original ENZYME format

All UniProtKB/Swiss-Prot entries referenced in this entry, with possibility to download in different formats, align etc.
All ENZYME / UniProtKB/Swiss-Prot entries corresponding to 3.4.11.-
All ENZYME / UniProtKB/Swiss-Prot entries corresponding to 3.4.-.-
All ENZYME / UniProtKB/Swiss-Prot entries corresponding to 3.-.-.-

USSN: 10/527,771
Attorney Docket: 1-2002.015 US

EXHIBIT B

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Molecular Cloning of an Insect Aminopeptidase N That Serves as a Receptor for *Bacillus thuringiensis* CryIA(c) Toxin*

(Received for publication, April 26, 1995)

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The *Bacillus thuringiensis* CryIA(c) insecticidal δ-endotoxin binds to a 120-kDa glycoprotein receptor in the larval midgut epithelia of the susceptible insect *Manduca sexta*. This glycoprotein has recently been purified and identified as aminopeptidase N. We now report the cloning of aminopeptidase N from a *M. sexta* midgut cDNA library. Two overlapping clones were isolated, and their combined 3095-nucleotide sequence contains an open reading frame encoding a 990-residue prepro-protein. The N-terminal amino acid sequence derived from the glycoprotein is present in the open reading frame, immediately following a predicted cleavable signal peptide and a pro-peptide. There are four potential *N*-linked glycosylation sites. The C-terminal sequence contains a possible glycosylphosphatidylinositol (GPI) anchor signal peptide, which suggests that, unlike most other characterized aminopeptidases, the lepidopteran enzyme is anchored in the membrane by a GPI anchor. This was confirmed by partial release of aminopeptidase N activity from *M. sexta* midgut brush border membranes by phosphatidylinositol-specific phospholipase C. The deduced amino acid sequence shows significant similarity to the zinc-dependent aminopeptidase gene family, particularly in the region surrounding the consensus zinc-binding motif characteristic of these enzymes.

The target of insecticidal *Bacillus thuringiensis* crystal δ-endotoxins is the apical (brush border) membrane of larval midgut cells (1). *In vitro* binding assays have demonstrated that the CryIA(c) toxin binds specifically and with high affinity to a single receptor species in brush border membranes prepared from larvae of the susceptible lepidopteran, *Manduca sexta* (2). Ligand blotting experiments have identified a single 120-kDa toxin-binding glycoprotein in *M. sexta* larval midgut membranes as the most likely candidate for the cellular CryIA(c) receptor (3, 4).

We recently reported the purification of this 120-kDa putative receptor from *M. sexta* midgut membranes by a combination of protoxin affinity chromatography and anion-exchange chromatography (5). N-terminal and internal partial amino acid sequences were similar to sequences of the ectoenzyme aminopeptidase N, and the purified 120-kDa glycoprotein displayed aminopeptidase N but not alkaline phosphatase activity. CryIA(c) toxin itself had no apparent effect on aminopeptidase activity over a range of concentrations. In ligand blotting experiments, the purified glycoprotein had the characteristics predicted of the receptor; it bound CryIA(c) toxin in the presence of GlcNAc but not GalNAc, it bound the lectin SBA, but it did not bind CryIB toxin (Ref. 5 and references therein).

The same glycoprotein was partially purified by Sangadala *et al.* (6) who used isoelectric focusing and immunoaffinity chromatography to obtain a mixture of 120- and 65-kDa midgut brush border proteins from *M. sexta*. Both (glyco)proteins bound CryIA(c) toxin in ligand blots, although the 120-kDa band was the major toxin-binding component (4). Enzyme assays revealed both aminopeptidase and alkaline phosphatase activity in the partially purified preparation, and the 120-kDa protein was identified as aminopeptidase N from the partial amino acid sequence. When reconstituted into phospholipid vesicles, the protein mixture increased toxin binding by 35% and enhanced toxin-induced $^{86}Rb^+$ release up to 1000-fold. This important result is the first (and so far only) demonstration that a partially purified receptor can potentiate the action of a toxin *in vitro*.

Aminopeptidase N (CD13; microsomal aminopeptidase; α-aminoacyl-peptide hydrolase (microsomal); EC 3.4.11.2) is a well documented zinc-dependent peptidase that catalyzes removal of N-terminal, preferentially neutral residues from peptides (reviewed in Ref. 7). This ectoenzyme is commonly found in the brush border membranes of the alimentary tract in a variety of different organisms. Recent reports have shown that a number of coronaviruses and a herpesvirus use aminopeptidase N as a receptor in their target tissue (8–10).

Following receptor binding at the midgut epithelium, toxins probably act by opening nonspecific channels or pores in the membrane, which leads to colloid osmotic lysis of midgut cells and ultimately the death of the insect (11). With the aim of understanding both the biochemical basis for toxin specificity and the mechanism(s) by which membrane insertion and cytolysis occur, we have cloned and sequenced the cDNA of *M. sexta* aminopeptidase N, a putative CryIA(c) receptor.

EXPERIMENTAL PROCEDURES

Polymerase Chain Reaction Amplification—PCRs[1] were performed by standard techniques (12). If the PCR product was to be sequenced, *Pfu* DNA polymerase (Stratagene) was used in the amplification be-

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The nucleotide sequence reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number X89081.

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[1] The abbreviations used are: PCR, polymerase chain reaction; BBMV, brush border membrane vesicles; bp, base pair(s); nt, nucleotide(s); pfu, plaque-forming units; PI-PLC, phosphatidylinositol-specific phospholipase C; GPI, glycosylphosphatidylinositol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CMC, critical micellar concentration.

cause of its high fidelity. Otherwise, *Taq* DNA Polymerase (Promega) was used in all PCRs. Single-stranded cDNA from *M. sexta* midgut brush border membranes was prepared as described previously (5).

Oligonucleotide Synthesis and Labeling—Oligonucleotides used as PCR primers and hybridization probes were synthesized on a Millipore Expedite 8909 nucleic acid synthesizer. Oligonucleotide probes were 3′ end-labeled with digoxigenin using the digoxigenin oligonucleotide tailing kit from Boehringer Mannheim and were used according to the manufacturer's recommendations.

cDNA Cloning and Sequencing—The *M. sexta* midgut brush border membrane cDNA library in λgt10 was a gift from Dr. J. Van Rie, Plant Genetic Systems, Belgium. The library was screened by the PCR-based microtiter plate technique described by Israel (13). Briefly, 8000 pfu arranged at 125 pfu/well in an 8 × 8 well array were screened by PCR (primers 3F and 5R). Two wells tested positive, and the phage from these were titered and rescreened at 4 pfu/well. One PCR-positive well from the secondary screen was selected, individual phage clones were plaque-purified, and phage DNA was prepared by the plate lysis method (12). Their identity as aminopeptidase N clones was confirmed by Southern blotting.

Subcloning and DNA Sequence Analysis—The λAPN cDNA insert was excised from the phage vector with *Eco*RI and subcloned into *Eco*RI-cut pBluescript II SK(−) (Stratagene). The λ5′APN blunt-ended PCR product was subcloned into *Eco*RV-cut pBluescript II SK(−), and the phage vector sequence was excised on a *Bam*HI fragment. All subcloning operations were performed by standard techniques (12). DNA was sequenced on an Applied Biosystems Inc. 373 automated DNA sequencer, using an Applied Biosystems Inc. Dye-Deoxy Terminator Cycle sequencing kit. A double-stranded nested deletion kit (Pharmacia Biotech Inc.) was used to generate a set of progressively smaller subclones of λAPN for sequencing. All clones were completely sequenced on both strands. DNA and protein sequences were assembled and analyzed using the Genetics Computer Group program package (14) and the Lasergene package (DNASTAR).

PI PLC Digestions and Aminopeptidase N Assay—*M. sexta* brush border membrane vesicles, prepared as described (15), were suspended at 2 mg/ml in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, pH 7.4). PI-PLC from *Bacillus cereus* (Sigma) was added at a final concentration of 2 units/ml and incubated for 90 min at 30 °C. The vesicles were pelleted by centrifugation at 13,000 × *g* for 10 min, the pellet resuspended in the same volume of phosphate-buffered saline, and the supernatant and pellet assayed for aminopeptidase and alkaline phosphatase activity as described (5). Control release was measured under the same conditions in the absence of PI-PLC. Release by detergents was carried out by the same method, using final concentrations of 0.1% (v/v) Triton X-100 or 0.5% (w/v) CHAPS

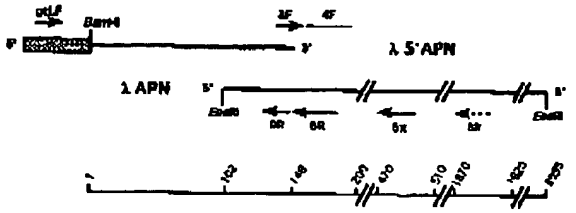
RESULTS

Partial Amino Acid Sequence and PCR—Following purification of aminopeptidase N from *M. sexta* midgut epithelium, both N-terminal and internal partial amino acid sequences were obtained from the glycoprotein. A possible overlap between the N-terminal sequence and internal amino acid sequence 77 (5) was tested by nested PCR using fully degenerate primers. When subcloned and sequenced, the cDNA sequence confirmed the overlap between the two partial amino acid sequences and also yielded 45 bp of unambiguous aminopeptidase N gene sequence (nt 139–183 in Fig. 2), which was used to design a unique forward PCR primer 3F and an oligonucleotide probe 4F (Fig. 1).

A fully degenerate antisense reverse PCR primer 3R was designed from internal amino acid sequence 68.5, QIVDDVF (5). This primer was used in conjunction with forward primer 3F to amplify fragments of aminopeptidase N cDNA from *M. sexta* midgut single-stranded cDNA preparations (Fig. 1). A single 1700-bp PCR product was identified by hybridization with 4F and was gel-purified and directly sequenced. This unambiguous gene sequence was used to design a unique reverse PCR primer 5R (see Fig. 1), situated 345 bp downstream of the unique forward PCR primer 3F.

Isolation of Two Overlapping Clones for Aminopeptidase N—The unique primer pair 3F/5R was used to screen a *M. sexta* midgut cDNA library in λgt10 using the high stringency PCR-based technique of Israel (13). 8000 phage clones were screened, and one positive recombinant phage, λAPN, was obtained. Although the 2904-bp cDNA insert (nt 102-3095 in Fig. 2) was found to contain an open reading frame that encoded the N terminus and all eight tryptic peptides derived from the purified protein (5), no initiating ATG codon was found, indicating that clone λAPN does not contain the total mRNA sequence. Attempts to obtain the missing 5′ end of the mRNA by 5′-rapid amplification of cDNA ends (16) were unsuccessful, and therefore the cDNA library was screened again by nested PCR, using a forward primer (gtLF) sited in λgt10 and two nested reverse primers (8R and 9R) at the 5′ end of clone λAPN (see Fig. 1). A single 350-bp PCR product, λ5′APN, was obtained containing 148 bp of aminopeptidase N cDNA (nt 1–148 in Fig. 2), including a 47-bp overlap with the 5′ end of λAPN. The new 5′ cDNA still did not contain an initiating ATG codon, but it did encode a putative N-terminal cleavable signal peptide (see below).

A



B

Name	Oligonucleotide sequence (5′ - 3′)	Position in cDNA (Fig.2)
3F	AGACATTACGCTGTGAC	140 - 156
4F	CCTGACTCCATACTTTGACGTCGTACC	157 - 183
5R	TTCTATCGACGTACCAACTTCTGTAGAACCC	513 - 482
8R	GTCAAAGTATGGAGTCAGGGTCACAGC	149 - 175
9R	GTAATGTCTTGGCCGGGTGCTAGTAG	129 - 148
gtLF	GCTCAACGTGCCAACAAATCTAAC	-

FIG. 1. **Aminopeptidase clones and PCR primers.** *A*, relationship between λ5′APN (*top*) and λAPN (*bottom*) and location of oligonucleotides used as PCR primers (*arrows*) and hybridization probes (*lines*). Primer 3R (*broken line*) is a fully degenerate oligonucleotide predicted from partial amino acid sequence, while all other oligonucleotides are designed from a unique cDNA sequence. The scale refers to the position in the combined cDNA sequence (Fig. 2). *B*, sequence of unique oligonucleotides used in PCR amplifications

Nucleotide and Deduced Amino Acid Sequence—Both λAPN and λ5′APN cDNAs were subcloned and sequenced on both DNA strands as described under "Experimental Procedures." The combined 3095-bp nucleotide sequence (Fig. 2) has an in-frame ATG codon at the 5′ end of the cDNA (nt 94–96), but this is probably not a start codon since it does not meet the criteria for a Kozak consensus translational initiation site (17). Therefore, the combined cDNA sequence is presumed to be missing a 5′ upstream sequence, including the initiating ATG codon. There is a long open reading frame starting at nucleotide 2 and extending 2970 bp to a TAA stop codon at nucleotide 2971. The short 124-bp 3′ noncoding region includes two additional in-frame stop codons and two consensus AATAAA polyadenylation signals contained within a 17-bp repeat (nt 2989–3006 and nt 3064–3081, Fig. 2), which may imply the occurrence of polymorphism in the 3′ noncoding region of the mRNA.

The 2970-bp open reading frame encodes a protein of 990 residues (Fig. 2). The N-terminal sequence of the mature (pu-

B. thuringiensis CryIA(c) Toxin Receptor 17767

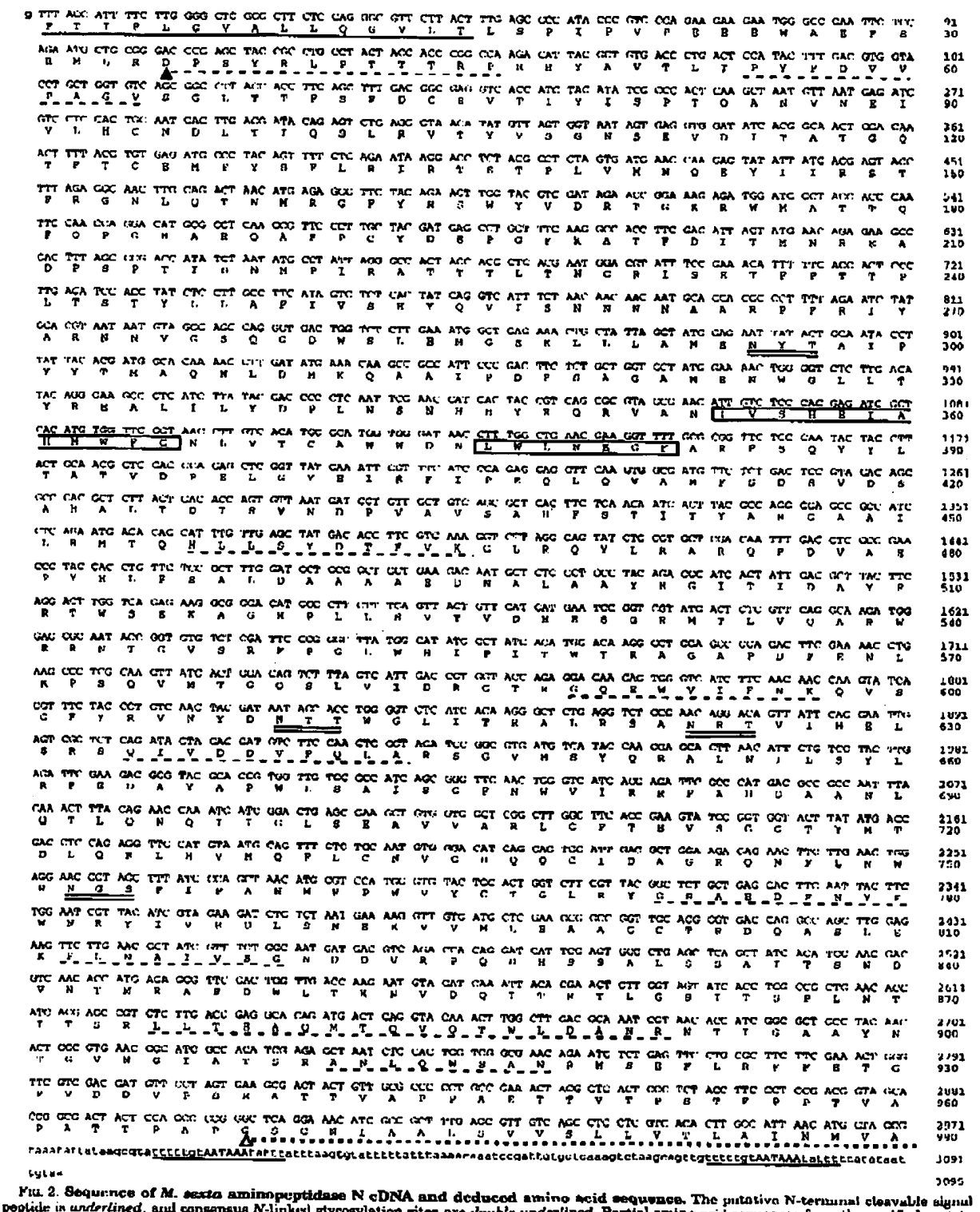


FIG. 2. **Sequence of *M. sexta* aminopeptidase N cDNA and deduced amino acid sequence.** The putative N-terminal cleavable signal peptide is *underlined*, and consensus *N*-linked glycosylation sites are *double-underlined*. Partial amino acid sequences from the purified protein are *broken underlined*, and the N-terminal residue of the mature protein determined by Edman degradation is designated by a ▲. The zinc binding/catalytic site (gluzincin motif) is *boxed*. The GPI signal peptide is *dot-underlined*, and Δ indicates the probable cleavage/attachment site of the anchor moiety. Repeats in the 3′-untranslated tail are *underlined*, and the two polyadenylation signals are in *capitals*.

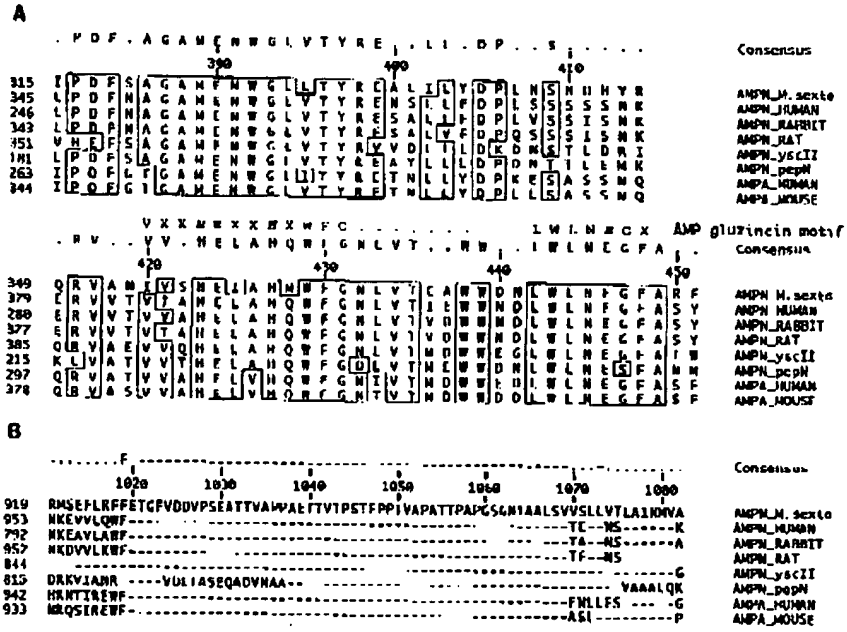


FIG. 3. **Aminopeptidase sequence alignment.** Alignment of the deduced amino acid sequences of aminopeptidase N from *M. sexta* with aminopeptidase N from human (18), rabbit (19), and rat (20), aminopeptidase yscII from *S. cerevisiae* (21), alanine aminopeptidase (*pepN*) from *L. delbrueckii* (22), and the aminopeptidase A from human (24) and mouse (23). *Letters* in the consensus sequence represent residues common to all sequences. *Numbers* to the *left* refer to the first residue in each line relative to the start codon of each respective primary sequence. *A*, highly conserved block including the zinc binding/catalytic site typical of the aminopeptidase family of gluzincins. Conserved sequences are *boxed*. The gluzincin motif is shown above the consensus sequence, with catalytic residues in *bold face* and zinc binding ligands in *bold/face italics*. *B*, *M. sexta* aminopeptidase N C-terminal extension containing the GPI signal peptide not found in other aminopeptidases.

rified) protein determined by Edman degradation extends from Asp^{66} to Pro^{44} (Fig. 2). The presence of residues upstream of Asp^{66} suggests that in *M. sexta*, aminopeptidase N is synthesized as a larger precursor protein and is trimmed to a mature product by limited proteolysis. There are four consensus Asn-*X*-Ser/Thr sequences, indicating possible *N*-glycosylation sites in the protein.

Homology to Other Aminopeptidases—Searches of the SwissProt (EMBL) protein sequence data base with the primary structure of *M. sexta* aminopeptidase N showed significant similarity to human (31% identity) (18), rabbit (31% identity) (19), and rat aminopeptidase N (31% identity) (20), to aminopeptidase yscII from *Saccharomyces cerevisiae* (29% identity) (21), alanine aminopeptidase (*pepN*) from lactobacterium (27% identity) (22), and also to mouse (29% identity) (23) and human (28% identity) (24) aminopeptidase A. A multiple sequence alignment between these known aminopeptidase sequences and the *M. sexta* sequence showed that the most striking similarity was around the characteristic and functionally crucial zinc-binding motif between residues Ile^{364} and Phe^{382} (Fig. 3*A*). This sequence classifies the *M. sexta* protein as a member of the aminopeptidase family of gluzincins, with His^{367}, His^{361}, and Glu^{385} being zinc ligands and Glu^{858} being involved in catalysis (25). An obvious difference in the alignment was the C-terminal 40–60-residue extension of the *M. sexta* sequence, which includes the GPI anchor signal peptide (Fig. 3*B*; see below). This feature probably reflects the fact that other membrane-bound aminopeptidases are generally anchored by an N-terminal signal anchor sequence.

Membrane Anchoring—In the epithelial cells of mammalian kidney and intestine aminopeptidase N is a type II membrane protein, anchored by an uncleaved N-terminal signal anchor sequence and with a C-terminal extracellular domain (26, 27). However, treatment of *M. sexta* brush border membrane vesicles (BBMV) with proteinase K leads to the release into the supernatant of a 100-kDa soluble form of aminopeptidase N, with the same N-terminal amino acid sequence as the membrane-bound form of the protein (data not shown). This suggests that the *M. sexta* aminopeptidase N is a type I membrane protein, anchored in the membrane by a C-terminal "stop-transfer" sequence and with an N-terminal extracellular domain. Such a topology would require an N-terminal cleavable signal peptide to initiate translocation across the endoplasmic reticulum membrane (27).

A hydropathy plot of the predicted primary structure (Fig. 4*A*) reveals one region at the N terminus and one at the C terminus with hydropathy averages greater than 1.6 and thus capable of spanning the membrane in a helical conformation (28). Although there is a third and comparatively shorter hydrophobic region centered around Ala^{248}, biochemical analysis of the protein (preceding paragraph) indicates that it is unlikely to be a transmembrane helix. Analysis of the N-terminal hydrophobic region by the weight-matrix method of von Heijne (29) for predicting signal sequence cleavage sites yields a "score" of 7.8 for cleavage after residue 15, while all other residues give scores of 1.9 or less. Known cleavage sites in other proteins typically have scores of 6–12. The algorithm gives a correct prediction in 75–80% of cases (29), and on this evidence it seems probable that the N terminus of the deduced polypeptide is a cleavable signal sequence, with scission by the signal peptidase occurring on the C-terminal side of Thr^{15}. According to this predicted topology, the sequence between Thr^{15} and Asp^{25} (the N terminus of the mature protein as determined by Edman degradation) constitutes a propeptide, the proteolytic release of which might serve to activate pro-aminopeptidase N. Although activation propeptides are a common feature of many proteases, hormones, and growth factors (reviewed in Ref. 30), there is only one other example of a putative propeptide region in an aminopeptidase, predicted from the cDNA-derived primary structure of aminopeptidase Y in *S. cerevisiae* (31).

A closer examination of the C-terminal hydrophobic sequence suggests that it is not the stop-transfer sequence of a type I membrane protein (27) since it lacks charged residues flanking the hydrophobic region, particularly positive charge at the C terminus typically found in such sequences (32–34). However, it does show the characteristics of a signal peptide for the addition of a glycosylphosphatidylinositol (GPI) anchor: a C-terminal run of 19 hydrophobic residues (Ile^{922}–Ala^{940}), pre-

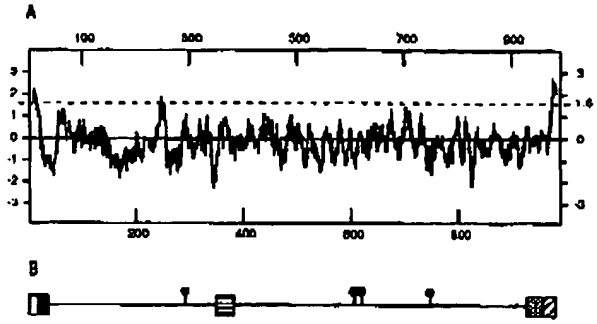


FIG. 4. *A*, hydropathy plot of the *M. sexta* aminopeptidase N protein sequence. The method of Kyte and Doolittle (28) was used with averaging over a window of 11 residues. Hydrophobicity resulted in positive and hydrophilicity in negative values. *B*, schematic diagram of *M. sexta* aminopeptidase N protein sequence. The predicted N-terminal cleavable signal peptide (*open box*) is followed by a predicted pro-peptide (*filled box*). The *horizontally hatched box* represents the gluzincin motif, while at the C terminus there is a predicted *O*-glycosylated stalk (*dotted box*) and the GPI signal peptide (*diagonally hatched box*). The four potential *N*-linked glycosylation sites are indicated as *knobs*.

coded by a cluster of three small residues (Gly^{968}–Gly^{970}, see Fig. 2), which functions as a cleavage/attachment site (35, 36).

A common diagnostic test for a GPI-anchored protein (37) is to demonstrate its release from the membrane by bacterial PI-PLC. Following incubation of *M. sexta* BBMV with PI-PLC, 16.0 ± 0.4% of total aminopeptidase N activity was released into the supernatant ($n = 4$) compared with a release of 4.8 ± 0.9% in the absence of PI-PLC ($n = 7$). In comparison, PI-PLC released 83.5 ± 2.6% of alkaline phosphatase activity into the supernatant ($n = 4$), compared with 9.9 ± 3.2% release in the control ($n = 4$). Differential solubilization by detergents can also be used to predict the presence of a GPI membrane anchor (38, 39), since only detergents with a high critical micellar concentration (CMC) are able to release significant amounts of GPI-anchored ectoenzymes into the supernatant. Treatment of *M. sexta* BBMV with 0.5% CHAPS (high CMC) released 78% of the total aminopeptidase N activity into the supernatant ($n = 2$), while 0.1% Triton X-100 (low CMC) released only 7% of total activity ($n = 1$). Although PI-PLC releases only a fraction of the total aminopeptidase N activity into the supernatant, this result demonstrates that at least a proportion of the *M. sexta* enzyme is linked to the brush border membrane by a GPI anchor. A similar study (40) showed that aminopeptidase N in the brush border membrane of the closely related lepidopteran *Bombyx mori* is also GPI-anchored. PI-PLC caused a maximal 40% release of *B. mori* aminopeptidase N activity compared with a 90% release of alkaline phosphatase activity.

DISCUSSION

In this study, partial amino acid sequence from aminopeptidase N purified from *M. sexta* midgut epithelium as a putative *B. thuringiensis* CryIA(c) toxin receptor was used to isolate *M. sexta* midgut aminopeptidase N cDNA clones by a PCR-based approach. Analysis of the 990-residue deduced amino acid sequence indicates that it is a large prepro-protein (Fig. 4*B*). The two pre-regions are the C-terminal GPI signal sequence (residues 968–990) and the (predicted) N-terminal cleavable signal sequence (residues 1–15), while the sequence between the predicted signal peptidase cleavage site and the N terminus of the mature protein (residues 16–35) is presumably a pro-region. Following proteolytic release of these pre- and pro-sequences, the mature polypeptide would then be 934 residues long, with a calculated molecular mass of 105 kDa. A 33-amino-acid long region (residues 935–967) immediately preceding the GPI signal peptide is rich in serine and threonine residues, which are potential *O*-glycosylation sites, and also in the helix-breaking amino acid proline, commonly found in β-turns. By analogy to decay accelerating factor, sucrase/isomaltase, low density lipoprotein receptor, and the mucin protein family (reviewed in Ref. 41), this region may represent a rigid, *O*-glycosylated stalk that serves to elevate the active site of the enzyme well above the cell surface. The mature protein sequence also has four consensus *N*-glycosylation sites, and lectin binding studies have indicated that at least one of these sites is occupied.[2] The presence of covalently attached carbohydrate may explain the observed difference between the molecular mass of the purified enzyme (120 kDa) and that of the polypeptide predicted from cDNA sequence (105 kDa).

A number of ectoenzymes are now known to possess GPI membrane anchors including acetylcholinesterase, alkaline phosphatase, microsomal dipeptidase, 5′-nucleotidase, trehalase, and aminopeptidase P in mammals (reviewed in Refs. 42 and 43) and alkaline phosphatase (44) and aminopeptidase N (40) in the midgut of the lepidopteran larva *B. mori*. It is common to find that treatment of ectoenzymes with PI-PLC releases only a fraction of the total activity. This observation implies that the uncleaved enzyme population is either anchored by a modified GPI structure that is insensitive to PI-PLC (reviewed in Ref. 36) or by a conventional C-terminal hydrophobic amino acid sequence that arises by alternative splicing of a single mRNA transcript, as is known to be the case with neural cell adhesion molecules (46). Although *M. sexta* aminopeptidase N activity is relatively resistant to PI-PLC release, this latter explanation seems unlikely since Northern blot analysis indicates that there is only one aminopeptidase N transcript in *M. sexta* midgut mRNA preparations.[3] Therefore the relative resistance of *M. sexta* aminopeptidase N to PI-PLC cleavage is probably due to modification to the GPI anchor structure itself.

In addition to its role as a receptor for *B. thuringiensis* CryIA(c) toxin, aminopeptidase N is known to be commandeered as a receptor by human (9) and porcine (8) coronaviruses, and by a human herpesvirus (10). In the latter two cases, studies demonstrated that the catalytic site and the viral binding site were on different domains and that aminopeptidase enzyme activity was not necessary for viral infection (10, 47). In our hands CryIA(c) toxin has no effect upon aminopeptidase N activity, which suggests that, like the viruses, the toxin binds at a site distinct from the catalytic site. As an exopeptidase, aminopeptidase N cannot itself be involved in the proteolytic activation (48, 49) of the 133-kDa CryIA(c) protoxin to the 65-kDa active toxin. Nor could it be responsible for cleavage of loop regions within the active toxin (50), although in theory the enzyme could contribute to any N-terminal trimming reactions following endoprotease cleavage. Thus, it seems that the feature of aminopeptidase N being exploited by both the viruses and CryIA(c) is simply its abundance at the apical membrane of epithelial cells, irrespective of its function as a protease. This does not preclude the possibility that following binding to the aminopeptidase receptor, CryIA(c) toxin may subsequently interact with other membrane components to which aminopeptidase N is functionally linked.

Vadlamudi *et al.* (51) identified (and subsequently purified) a 210-kDa putative CryIA(b) receptor in the brush border membrane of *M. sexta*. The same authors (45) recently reported the cloning from *M. sexta* of this putative CryIA(b) receptor. The cDNA clone encodes a novel cadherin-like glycoprotein which,

[2] P. J. K. Knight, unpublished data.
[3] J. C. Martinez, unpublished data.

when expressed in either COS-7 or human embryonic 293 cells, was able to bind CryIA(b) toxin in ligand blotting experiments and in the latter case also in homologous binding assays.

The demonstration (6) that partially purified aminopeptidase, when incorporated in liposomes, requires dramatically less CryIA(c) toxin to induce a given amount of $^{86}Rb^+$ leakage compared with vesicles containing no brush border membrane protein strongly suggests that the 120-kDa aminopeptidase N glycoprotein functions as a CryIA(c) receptor *in vivo*. Having cloned *M. sexta* aminopeptidase N, we are in a position to directly investigate its interaction with CryIA(c) toxin.

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